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Purification and Characterization of a Fibrinolytic Protease from *Bacillus subtilis* using Blood Fibrin of *Bos taurus* and *Ovis aries*

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ABSTRACT:
A fibrinolytic protease from two different blood fibrin *Bos taurus* and *Ovis aries* as an added ingredient by *Bacillus subtilis* which was purified by ammonium sulphate precipitation, dialysis and ion exchange chromatography. The purification protocol resulted in 1.063 and 1.190-fold purification of the enzyme. The apparent molecular weight of the enzyme was found to be 30.0 kDa, determined by sodium dodecylsulphate poly acrylamide gel electrophoresis. Optimal temperature and pH were found to be 37°C and 6.0 respectively. Upon the addition of zinc chloride the enzyme activity was increased while EDTA and PMSF inhibited the activity of the enzyme, indicating the presence of metalloprotease and serine protease. The produced enzyme showcases the blood clot-busting activity.

Key Words: Fibrinolytic protease, *Bos taurus*, *Ovis aries*, *Bacillus subtilis*

INTRODUCTION

Cardiovascular diseases such as acute myocardial infarction, ischemic heart disease, and high blood pressure are the leading causes of death in the world [1]. Among the different types of cardiovascular diseases, thrombosis is one of the most widely occurring diseases in modern life. Drugs using fibrinolytic enzymes are the most effective methods in the treatment of thrombosis. Fibrin is the major protein component of blood clots, which is formed from fibrinogen by thrombin (EC 3.4.21.5). The insoluble fibrin fiber is hydrolyzed into fibrin degradation products by plasmin (EC 3.4.21.7), which is activated from plasminogen by tissue plasminogen activator (tPA). Although fibrin clot formation and fibrinolysis are maintained in balance by the biological system, thromboses, such as myocardial infarction, occur when clots are not lysed as a result of a disorder of the balance [2]. In the unbalanced state, the clots are not lysed and thus, thrombosis occurs. tPA has been developed for the treatment of thrombosis because of its efficacy and stronger affinity to fibrin. Intravenous administration of thrombolytic agents; lumbrokinase (EC 3.4.17.13) urokinase and streptokinase has been widely used for thrombosis therapy and their major functions have been described as plasminogen activators or plasmin-like proteases which can directly degrade fibrin or fibrinogen, thereby dissolving thrombi rapidly and completely, but these enzymes have a low specificity to fibrin and are expensive [3,4].

Bacillus sp. produces a variety of extracellular and intracellular proteases. An alkaline protease (subtilisin), a neutral metalloprotease, and an esterase are secreted into media, whereas at least two intracellular serine proteases are produced within *Bacillus* sp. [3,5-9]. In particular, the production of subtilisin protease has been exploited commercially for use in laundry detergents and for other applications [10,11].

Nattokinase (NK) (formerly designated Subtilisin NAT) [12] is an extracellular enzyme secreted by *B. subtilis* natto [13] and belongs to the alkaline serine protease family, the catalytic center of which contains three conserved residues, Asp-32, His-64, and Ser-221 [14]. It has a molecular mass of 27.7 kDa and an isoelectric point of 8.7 [15]. Nattokinase is composed of 275 amino acids, and the gene sequence is homologous to those of other members of the subtilisin family (99.5% homology with subtilisin E, 86% with subtilisin BPN′, and 72% with subtilisin Carlsberg) [16]. It not only degrades fibrin in thrombi [17] but also cleaves plasminogen activator inhibitor type I [17,18]. Nattokinase has greater thrombolytic activity than plasmin [15,17-19] a natural thrombolytic protease in blood, and increases the production of plasmin from plasminogen due to its action on plasminogen activator. These observations, together with the fact that it can be absorbed across the intestinal tract after oral administration [20,21] and induce fibrinolysis [21], make nattokinase a potential clot-dissolving agent for the treatment of cardiovascular disease. Dietary supplementation with natto suppresses the intimal thickening of arteries and leads to the lysis of mural thrombi seen after endothelial injury [22]. The use of oral administration of nattokinase in fibrinolytic therapy for thrombosis

and the prevention of atherosclerosis is therefore of interest. Nattokinase is currently used as a nutrient supplement to improve circulation in the body and has amyloid degrading ability [13,21,22,23].

In this paper, we report the comparative studies on the production, purification and characterization of the fibrinolytic protease produced by *Bacillus subtilis* from the blood fibrin of *Bos taurus* and *Ovis aries.*

MATERIALS AND METHODS

Production of Crude Enzyme

The producer microorganism were isolated from soil samples collected from a burial ground near Chennai, India and were characterized as *Bacillus subtilis* using morphological, physiological properties and biochemical tests [24,25] and maintained on an NB slant at 4°C. The production of crude enzyme were performed in 250 ml Erlenmeyer flasks, each containing 50 ml of protease production medium [composition (%w/v): glucose, 1.0 and peptone, 5.5; magnesium sulphate, 0.2; pH 7.5]. The substrate blood fibrin (0.6%) (From *Bos taurus* and *Ovis aries* blood samples) was added after sterilization aseptically. For the seed culture, one colony per plate was inoculated into 5 ml of production medium and incubated at 37°C in an orbital incubator shaker for 16 hours. The seed culture was inoculated 1% (v/v) in both the media and incubated at 37°C for 2 days.

Estimation of Crude Enzyme and Proteolytic Activity

The cell free supernatant (crude enzyme) obtained by centrifugation at 10,000 rpm for 30 minutes at 4°C was assayed for its total protein concentration using BSA as the standard protein [26]. About 5 µl of crude enzyme were spotted on skimmed milk agar plates and then incubated at 37°C for overnight for observing proteolytic activity by detecting the presence of clear zones.

Measure of Protease Activity

The presence of protease was measured by GCZ method for that gelatine gel was prepared by dissolving, by heating 0.8% agarose and 1.5% gelatine in a liter of phosphate buffer maintained at pH 7.5 [27]. Gel was poured onto the glass slides and solidified. Gel puncher was used to punch the cups in the gel and the cell free supernatant of both the blood fibrin sources were loaded using btrophase (anticoagulant) as control. The gel was kept in the Petriplate containing wet cotton under overnight for diffusion at 37˚C.

At the end of the incubation period, the plates were flooded with previously prepared mercuric chloride (HgCl) in HCl solution .The mean diameters of the clear zones was used as a measure of protease activity.

Protease Assay

The enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5% casein solution in 0.1 M carbonate buffer (pH 9.0) and 0.1 ml of enzyme solution in the total volume of 2.1 ml. After incubation at 37°C for 5 minutes, the reaction was stopped by adding of 3.0 ml of 10% ice cold TCA (Trichloroacetic acid) and then centrifuged at 10,000 rpm for 5 minutes at 4°C. The A_{280} for the supernatant was measured and converted to the amount of tyrosine equivalent. One unit (U) of protease activity was defined as the amount of enzyme required to release one µmol of tyrosine in one minute by 1.0 ml of enzyme solution under the assay conditions. Enzyme assay was performed for the crude enzyme and after each step of purification. The enzyme activity is defined as the unit of enzyme released per minute per ml. The specific activity is the ratio between the enzyme activities to the protein concentration. The purification factor or purification fold is the ratio between the specific activities of purified extract to the specific activity of crude extract. The yield is the ratio of the product of total protein and purified extract to the total protein content in the crude extract.

Purification by Ammonium Sulphate Fractionation

The production media was centrifuged at 10,000 rpm for 12 minutes to obtain the supernatant. The supernatant after centrifugation was collected and the volume of it was measured. And accordingly 70% ammonium sulphate was measured in order to conduct the salting out procedure under ice cold condition on the magnetic stirrer until complete dissolving takes place then it was kept for overnight in the refrigerator. The pellet was collected and dissolved in 10 ml of 50 mM Tris HCl (pH 8.5) [28]. This solution contains the enzymes precipitated by ammonium sulphate.

Purification by Dialysis

The dialysis was carried out in activated dialysis bag (the dialysis membranes are activated by cutting out about 8 cm of the dialysis tube and place it in 100 ml of 2% sodium bicarbonate. 1.0 mM EDTA was added to chelate any metal ions and was boiled for 10 minutes and wash it in boiling distilled water) with the enzyme and sealed to avoid air bubbles. The bag was dipped in 500 ml beaker filled with water on a magnetic stirrer in ice cold condition for 8 hours. The water was changed frequently for every hour to avoid equilibration.

Purification by Ion- Exchange Chromatography (DEAE-Sephacryl Column)

The dialyzed sample was removed from the tubing and filtered through a 0.45µm filter and then applied to a packing column (DEAE- Sephacryl) previously equilibrated with 0.01M Tris HCl buffer (pH 7.2) slowly percolating large volume of buffer through

packed material. A sample of desalted enzyme preparation was loaded onto the column. Flow rate was controlled at 0.5 ml/min by 5 ml of fractions collected and analyzed for protein and enzyme activity.

Molecular Weight Determination

SDS-PAGE was performed at room temperature [29]. The distribution of protein on the gel plate was revealed by staining with Coomassie Brilliant Blue R250 and destained with destaining solution (methanol/ acetic acid/ water = 3:1:6). The molecular weight of the protease was determined by comparison of the migration distances of standard marker proteins consisting serum albumin (66.2 kDa), ovalbumin (45.0 kDa) [30], galactosidase (116.0 kDa), phosphorylase b (97.4 kDa), carbonic anhydrase (31.0 kDa), trypsin inhibitor (21.5 kDa) [31].

Characterization of Purified Enzyme

The stability of protease was studied under different parameters such as pH, temperature, varying substrate concentration and effectors by incubating the purified enzyme with varying pH ranging from 2.0-10.0 using carbonate buffer (0.1 M). The enzyme samples were added to 1 ml of the buffer and incubated at 37°C for 5 minutes and specific protease assay was carried out. The thermal stability of the enzyme was analyzed by incubating at 4, 37, 55 and 85°C for 5 minutes. The activators and inhibitors were known to affect different classes of proteases [32,33]. To determine whether the activity of protease could be affected, varying concentrations of these effectors (0.2-1.0M) zinc chloride, phenylmethylsulfonyl fluoride (PMSF) and ethylene diamine tetra acetic acid (EDTA) was added with extracellular supernatant and incubated at 37˚C for 5 minutes followed by enzyme assay that showed the residual activity.

Determination of Fibrinolytic Activity

Fibrinolytic activity of each solution was determined by both the plasminogen-free fibrin plate method and the plasminogen- rich fibrin plate method [34]. Plasminogen-free fibrin plates consisted of the fibrinogen solution [5 mg of human fibrinogen in 7 ml of 0.1 M barbital buffer (pH 7.8), 10 U of thrombin solution, and 7 ml of 10 g/L agarose] in petridishes (9 cm in diameter). Fibrin plates were heated at 80°C for 30 minutes to destroy other fibrinolytic factors. Plasminogen-rich fibrin plates contained 5 U plasminogen, in addition, and were not heated. To observe the fibrinolytic activity, 10 µl of enzyme solution was carefully dropped onto a fibrin plate and was incubated at 37°C for 18 h. The activity fibrinolytic enzyme was estimated by measuring the dimension of the clear zone on the fibrin plate and plotting a calibration curve based on urokinase standard solutions.

RESULTS AND DISCUSSION

Analysis of Proteolytic Effect and GCZ Method

The observed *Bacillus subtilis* growth in the skimmed milk agar plate revealed the proteolytic effect. It was found that the clear zone formed around the streaked area indicating the components utilization i.e., the highest protease activity and the protease production was dependent on the bacterial growth. The gelatine agar plate showed a diameter of about 0.6-1.5 cm with 10-100 µl of filtrate free (*Bos taurus* blood fibrin) supernatant and showed a diameter of 1.3-1.9 cm with 10-100 µl of the filtrate free (*Ovis aries* blood fibrin) supernatant. This indicates that the crude enzyme had high proteolytic effect and has good affinity towards both the blood fibrin sources.

Purification and Molecular Weight Determination Analysis

The protease enzyme from the two different medium by *Bacillus subtilis* was purified to electrophoretic homogeneity by a three-step process, and carried out at room temperature except for centrifugation and precipitation (at 4°C). It was purified by a combination of ammonium sulphate precipitation (70%), dialysis and subsequent DEAE- Sephacryl ion exchange chromatography to homogeneity, which yielded washed-out fraction with high activity. The supernatant of 48 h grown culture of *Bacillus subtilis* was used as the crude source of enzyme. The enzyme from *Bos taurus* and *Ovis aries* was purified to 1.063 and 1.19-fold, with a final yield of 37.01 and 40.36% respectively (as shown in Table 1). The culture supernatant of *Bacillus subtilis* had an initial specific activity of 670.37 U/ml and 739.13 U/ml and the final specific activity of 712.76 and 879.6 U/ml after purification for *Bos taurus* and *Ovis aries* respectively. It shows the purification will result in the increase in enzyme specific activity. At the final stage of purification, the recovery of the enzyme activity was rather low. The low yield of purified enzyme can be attributed to loss during ammonium sulphate precipitation as well as tight binding of protease to the hydrophobic column as a significant amount of enzyme activity never eluted from the DEAE-Sephacryl column. After the combination of various purification techniques, finally the eluted protein fractions from the two sources were subjected to SDS-PAGE. Only one band was observed in the purified sample and the molecular weight of purified enzyme was determined nearly to be 30.0 kDa.(Fig. 1).

Characterization of Protease

The effects of temperature on the activity of the purified protease from both the sources are shown in (Fig. 2). The enzyme showed the maximal enzyme activity at 37°C and stability throughout the temperature. The purified enzyme activity reached up to 57.5 and 62.5 U/ml from blood fibrin of *Bos taurus*

and *Ovis aries*. The enzyme showed the stability at 55°C resulting in the minimal decrease in the activity. There was a significant decrease with further increase or decrease in the temperature. This inactivation of enzyme shows the destruction of enzyme at higher temperature. The results showed that the protease obtained from both the sources appeared to be heat labile at temperature 37-55°C. The stability of the enzyme at high temperature suggested its usefulness in industrial applications. The pH stability of the enzyme was investigated in the range of pH 2.0–10.0 (Fig. 3). The optimum pH for the enzyme was found to be 6.0 for both the sources, suggesting the slight acidic nature, thereby measuring the residual activity after incubation at each pH for 5 minutes at 37°C. The enzyme was very stable at the pH 6.0 at 37°C. The enzyme activity was increasing to a larger extent till 4.0% of substrate concentration (Fig. 4) but found to be negligible changeover than that with enzyme activity for bacterial protease. After the protease enzyme had been incubated with amino acid side-chain modification reagents at 37°C for 5 minutes, the enzyme activity was almost completely inhibited by PMSF (Fig. 5) and EDTA (Fig. 6) even in minimal concentration (0.2 mM). It is evident that the complete inhibition of protease activity with respect to the PMSF indicating the purified protease belongs to the serine protease as the PMSF is the strong inhibitor against serine protease. With increasing amounts of inhibitor almost complete inhibition was obtained at some higher concentration. This observation suggested that the hydrosulfuryl and metal might be present in or near the active site of the enzyme. Thus the protease from *Bacillus subtilis* is the hydrosulfuryl-metalloprotease. Since metal ions are essential for the activity of the enzyme, here effect of the metallic activator Zn^{+} ion was tested and it showed an increase in the activity obtained from both the sources. Higher concentration of activator showed higher protease activity (Fig. 7). The studies of metal ions on protease activity suggested that there is strong interaction between metal ions and enzyme may have several metal binding sites.

Table 1: **Comparison of enzyme before and after purification**

Enzyme Production	Concentration of protein (mg/ml)	Enzyme activity (U/ml)	Enzyme specific activity (U/ml)	Purification factor	Yield (%)
Crude Enzyme					
Bos Taurus	0.540	362	670.37	1	100
Ovis aries	0.575	425	739.13	1	100
After $(NH_4)_2SO_4$ Fractionation					
Bos Taurus	0.374	258.33	690.72	1.03	71.36
Ovis aries	0.414	308.34	744.78	1.01	72.55
After Dialysis					
Bos Taurus	0.225	157.8	701.33	1.046	43.58
Ovis aries	0.252	226.1	754.86	1.021	44.75
After Ion Exchange					
Bos Taurus	0.188	134	712.76	1.063	37.01
Ovis aries	0.195	171.5	879.6	1.190	40.36

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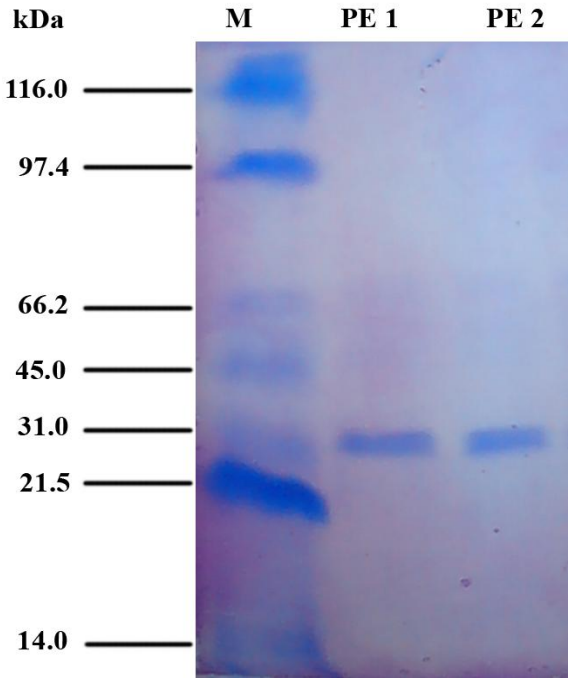

Fig. 1. SDS PAGE image for purified enzymes from different sources. PE 1- Purified enzyme 1(*Bos Taurus)*, PE 2 - Purified enzyme 2 (*Ovis aries)*

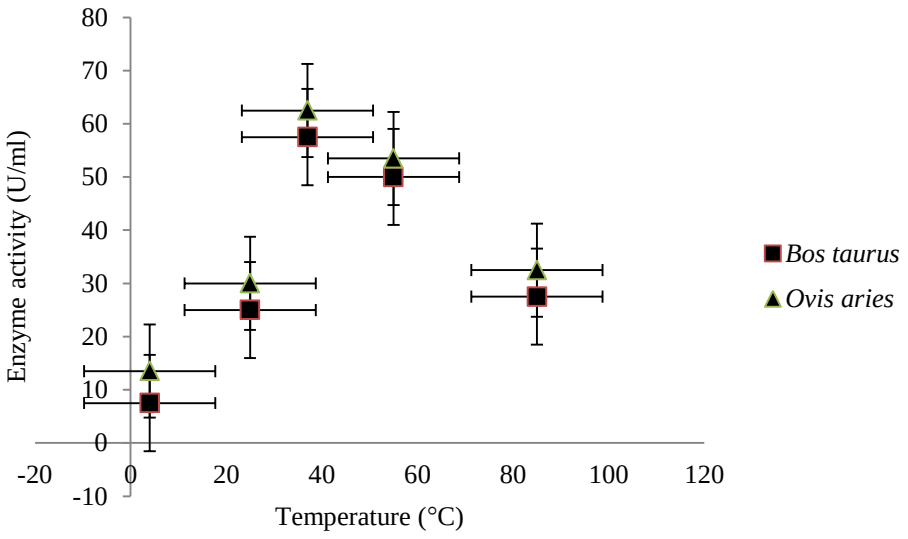

Fig. 2. Effect of temperature on enzyme activity

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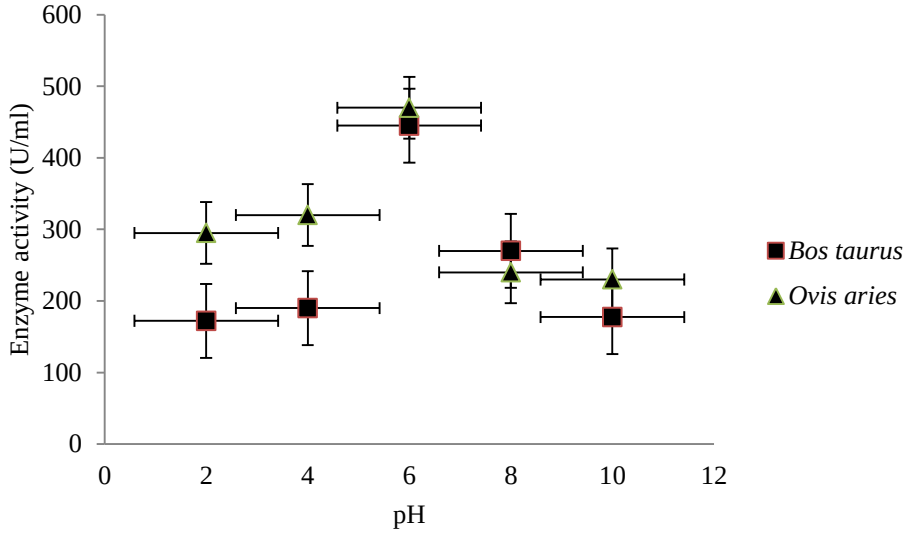

Fig. 3. Effect of pH on enzyme activity at 37˚C

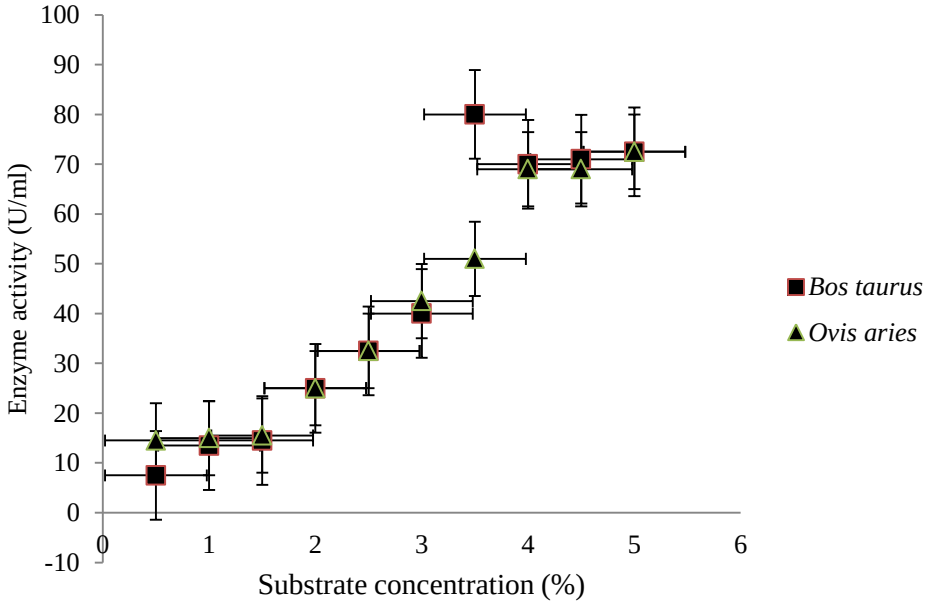

Fig. 4. Effect of substrate concentration on enzyme activity at 37˚C and pH 6.0

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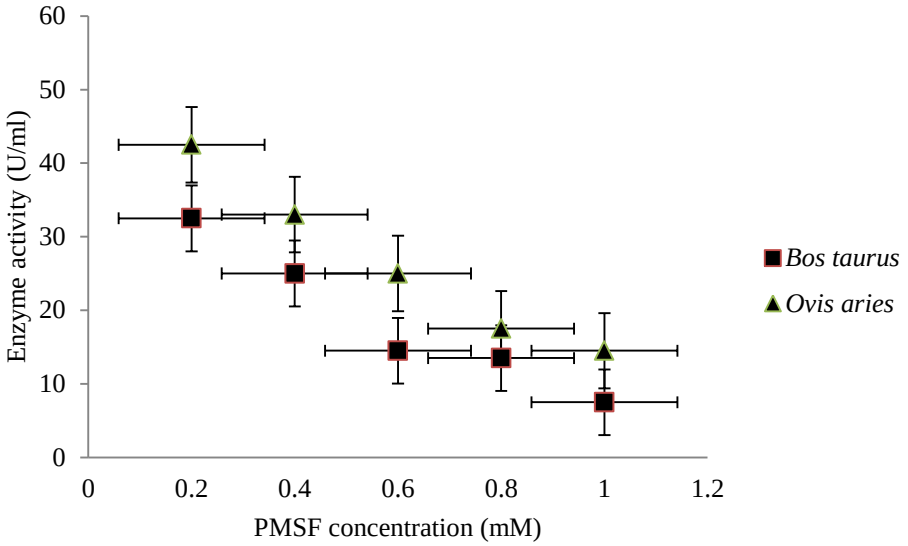

Fig. 5. Effect of PMSF on enzyme activity at 37˚C and pH 6.0

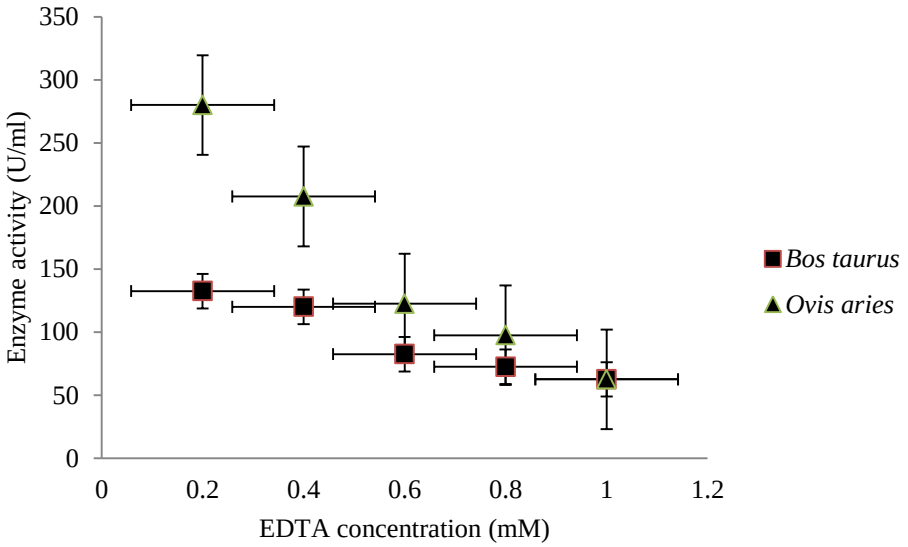

Fig. 6. Effect of EDTA on enzyme activity at 37˚C and pH 6.0

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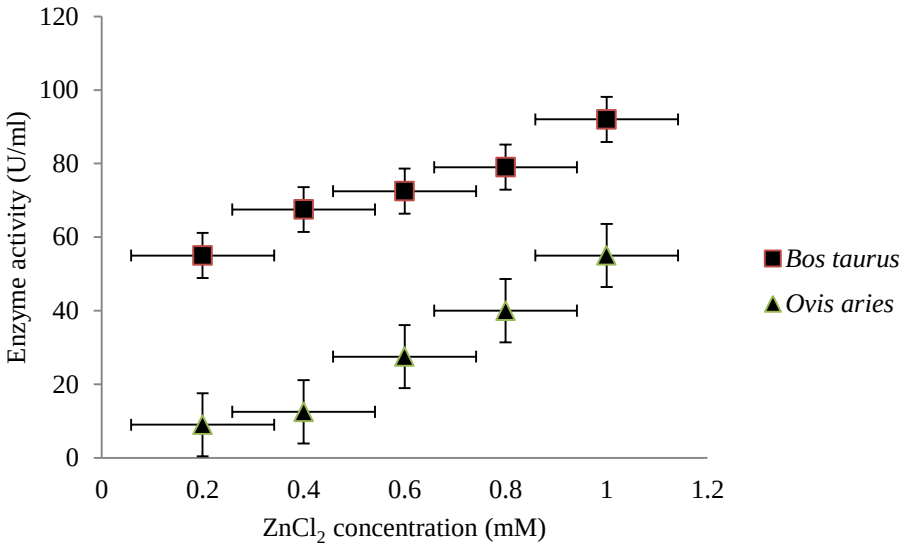

Fig. 7. Effect of zinc chloride on enzyme activity at 37˚C and pH 6.0

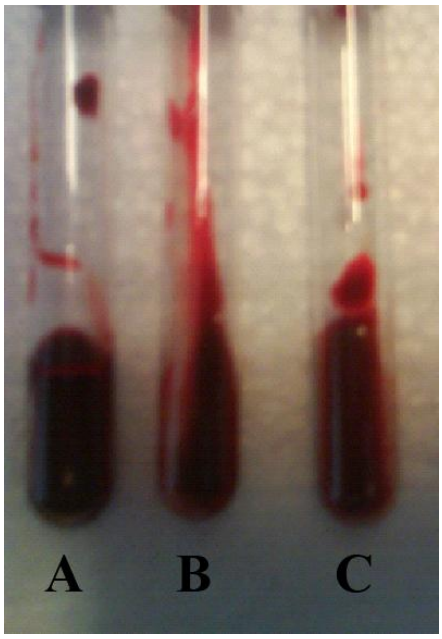

Fig. 8. Anti coagulant activity of protease
A – Without protease, B – With purified protease, C – With crude protease

Fibrinolytic Activity Analysis

The plasminogen was decomposed into the active fragment when they were loaded on to the fibrin plate. These results indicate that the enzyme is able to degrade fibrin clots in two ways, namely by forming active plasmin from plasminogen (plasminogen activator type), and by direct fibrinolysis. The produced enzyme is a fibrinolytic enzyme and differs from typical blood clotting enzymes such as thrombin, because fibrinogen was hydrolyzed, but no fibrin clot formed.

Fibrinolytic Protease as an Anti Coagulant

The produced fibrinolytic protease potentiates the clot busting activity. The normal clotting time for the blood is 5-10 minutes. When the purified enzyme was added to the blood sample, the time taken for the blood to clot became 6-7 minutes under optimum conditions and when crude enzyme was added to the blood samples, it took nearly 9 minutes for the blood to clot shown as A,B,C (Fig. 8). This indicates that the enzyme is able to degrade the blood clots in the absence of endogenous fibrinolytic factors. Thus the enzyme produced did have the property of an anti coagulant.

CONCLUSIONS

The enzyme which was produced from *Bacillus subtilis* using crude fibrin obtained from the blood samples of *Ovis aries* and *Bos taurus* was found to have characteristics same as that of serine protease as well as nattokinase. The enzyme thus produced was purified by DEAE Sephacryl Ion Exchange chromatography. The molecular weight was found nearly to be 30.0 KDa. The optimum temperature and pH for the enzyme produced from both the sources was found to be 37°C and 6.0 respectively. The activity of this enzyme increased with the addition of metal ions and decreased with addition of inhibitors such as EDTA and PMSF. The enzyme produced showed high activity but low concentration.

It can be concluded that the enzyme produced by *Bacillus subtilis* from *Ovis aries* is best option for the industrial production as compared to *Bos taurus*. The safety of the purified fibrinolytic protease, as an orally administered thrombolytic agent, its induction of in vivo lysis of the thrombi, and potential side effects need further research to confirm their physiological function.

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